Chapter 2

Characterization of Sinus Microbiota by 16S Sequencing from Swabs

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Abstract

New culture-independent microbiology methods are leading to a paradigm shift in our understanding of how the microbial community at the mucosal surface impacts sinonasal health and disease. Whereas traditional culture-based protocols were designed to identify specific pathogens in order to direct antibiotic therapies and eradicate bacteria, newer molecular techniques allow for the identification of both culturable and nonculturable bacteria in diverse communities. As a result of the recent explosion in the use of molecular techniques, we are gaining an understanding of how commensal bacteria may help modulate the host immune response and promote homeostasis. Here, we describe the general workflow of microbiome sequencing including the detailed methods for extracting mixed-community genomic DNA from sinonasal swabs, amplifying bacterial 16S rRNA genes using quantitative PCR, and preparing the samples for next-generation sequencing on the most commonly used sequencing platforms.

Key words Sinus swabs, DNA extraction, Bacterial 16S rRNA, Sinusitis, Culture-independent microbiology, Next generation sequencing, Microbiome

1 Introduction

The sinonasal cavity is known to harbor a rich and diverse variety of indigenous microbiota, which are increasingly appreciated for their role in promoting health and contributing to diseases such as acute and chronic rhinosinusitis [1–6]. Traditionally, the identification of microorganisms in the paranasal sinuses has relied on pure-culture techniques that often bias towards identifying more easily cultivated microorganisms and are limited in their capacity to identify particularly fastidious and/or anaerobic microbes [7]. Clinical microbiology has historically focused on identifying single pathogens that cause disease with the goal of targeting antibiotic therapy and eradicating bacteria. However, a new paradigm shift is underway emphasizing the role of commensal microbes within the community at the epithelial surface, and their ability to promote homeostasis and modulate the host mucosal immune response

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[8–10]. Recent breakthroughs in culture-independent molecular methods have increased our ability to identify and characterize both culturable and nonculturable microbial communities including fastidious and anaerobic microbes [11–13]. These molecular techniques have been used to profile the microbiome within the healthy paranasal sinuses, which were found to possess a rich and diverse community of microbes including representatives of the phyla Firmicutes, Proteobacteria, Actinobacteria, and Bacteroides. Frequently identified species from the sinonasal niche include *Staphylococcus epidermidis*, *S. aureus*, *Propionibacterium* and *Corynebacterium* spp. among others [14].

Bacterial 16S ribosomal RNA (16S rRNA) is a component of the 30S ribosomal subunit in prokaryotic ribosomes. The genes encoding for the 16S rRNA contain highly conserved nucleotide sequences that allow for the design of universal PCR primers, which can be used to amplify the total 16S rRNA genes within a sample in an "unbiased" fashion. In addition to these conserved regions, 16S rRNA genes contain hypervariable regions that reflect species-specific signatures of particular bacterial taxa. Since the 16S gene is highly conserved among bacteria, it has become the standard for identification and phylogenetic classification [15–17]. Although 16S rRNA sequencing is often recognized as "unbiased," this is a subject of debate since the universal bacterial 16S DNA primers target only the bacterial domain and therefore do not identify other prokaryotic domains such as archaebacteria and unclassified prokaryotes [18]. Since the advent of next generation sequencing techniques, several databases are now available that catalog specific type strains of 16S rRNA genes. These can be compared at: http://www.ncbi.nlm.nih.gov/genomes/static/16S_comparison_help.html.

Here we will describe the general workflow and in-depth methods for characterizing the microbiome by sequencing the 16S rRNA gene from sinonasal swabs. Swabs provide an efficient and minimally invasive means to sample this mucosal surface microbiome. Collected sinus swabs may contain mucus, blood, and epithelial cells in addition to resident microbes. This mixed-community genomic DNA is isolated and used as the starting material for microbiome sequencing.

There are several methods reported in the literature to extract mixed-community DNA from various tissues [19]. Commercially available DNA isolation kits work well for isolating DNA from samples with low biomass, such as sinus swabs. In our experience, the amount of biomass present on the swab following sample collection dictates which DNA extraction method will return the cleanest sample for gene amplification and subsequent sequencing. We have found that samples with low biomass and very little blood contamination can be isolated using a commercial kit or directly precipitated and extracted using an abbreviated boiling method in nonionic detergent (NP-40), whereas samples with higher biomass

including tissue and blood, are best extracted using a more rigorous phenol–chloroform solvent and grinding method [20–22].

Once DNA is isolated from other cellular components, the total bacterial 16S DNA in the sample is quantified by amplifying small-subunit (SSU) rRNA genes using fluorogenic universal primers and quantitative PCR (qPCR) to determine if there is bacterial DNA present in the sample and the optimal number of cycles for barcoding.

With sufficient quantities of 16S DNA confirmed, the individual samples are amplified again, this time using unique barcoding primers, which are primers containing short oligonucleotide sequences specific to one individual sample. These barcodes are also designed to contain a short nucleotide sequence, called an adapter, which is specific to the sequencing platform. Each of the major sequencing platforms—including those offered by Illumina, Nanion Technologies, Oxford Nanopore Technologies, and Pacific Biosystems—utilizes unique oligonucleotide adapter sequences for recognizing samples that must be incorporated when designing barcode primers. Several open source software tools are available for designing barcoding primers [23, 24].

After each sample is labeled with a unique molecular barcode, the samples are pooled, normalized for concentration, and then sequenced. Particular rDNA sequences identified within a swab sample that correspond to a clone in the rRNA gene reference library are considered to exist within the community sampled within the sinonasal mucosal niche. Relative abundance of various bacterial taxa within a sample can be deduced using species-specific DNA or RNA-hybridization probes.

16S microbiome sequencing is a rapidly evolving field with new technology emerging that promises to increase the number and length of sequences that are achievable in a given sequencing run. As a result, there are several platform specific nuances and methods for analyzing sequence data that are beyond the scope of this methods paper. Here we aim to provide a succinct and detailed description of isolating mixed-community genomic DNA from sinonasal swabs and preparing the DNA for microbiome sequencing. Significant expertise is required to continue beyond this point, and the reader is instructed to other references in terms of selecting the appropriate regions to sequence [25, 26], appropriate assessment of sequence quality [27], database alignment and statistical methods [28–31].

2 Materials

In order to reduce the risk of ribonuclease or bacterial DNA/RNA contamination, all buffers and aliquot reagents should be prepared within a HEPA filtered laminar flow PCR hood. Ensure that all surfaces are wiped clean with 70% ethanol and disinfect the hood

with 15 min of ultraviolet light before beginning. Whenever possible, use molecular grade reagents. Several of the listed reagents can be purchased premixed to limit potential for contamination.

2.1 Sample Collection

1. Single use, dry, sterile swabs. Several different swabs may be utilized, depending on the technique and desire. Flocked swabs can be utilized to obtain a more aggressive surface swab and incorporate host epithelial cells, if desired. We have utilized BD CultureSwab™ (Cat. No 220115) swabs to sample both awake and anesthetized patients, with sufficient tolerability and biomass yield to be able to sample subjects in a longitudinal fashion.

2.2 DNA Extraction from Low Biomass Swabs

1. Sterile 2 mL screw cap microcentrifuge tubes.
2. TEN Buffer: 10 mM Tris–HCl [pH 8.0], 1 mM EDTA, 1% NP40 nonionic detergent. Each sample requires 500 µL. For 100 mL, add 0.16 g Tris–HCL, 29.2 mg EDTA, and 1 mL NP40 to 99 mL of sterile molecular grade water.

2.3 DNA Extraction from High Biomass Swabs

1. Sterile 2 mL screw cap microcentrifuge tubes.
2. 2× Buffer B: 142.9 mM NaCl, 142.9 mM Tris–HCl [pH 8.0], 14.3 mM EDTA, 5.7% SDS. Each sample requires 700 µL. For 100 mL, combine 2.9 mL of 5 M NaCl stock, 14.3 mL 100× Tris–HCl stock, 418 mg EDTA, and 28.5 mL 20% SDS solution with 54.3 mL sterile molecular grade water.
3. Phenol–chloroform. Each sample requires 500 µL. For 100 mL, mix a 50 mL phenol and 50 mL chloroform.
4. Zirconia/Silica beads (0.1 mm): (BioSpec Cat. No 11079101z).
5. 7.5 M Ammonium acetate: Each sample requires 500 µL. For 100 mL, add 57.8 g ammonium acetate to 100 mL sterile molecular grade water.
6. Isopropanol (100%).
7. Glycogen (20 mg/mL): Each sample requires 2 µL. For 1 mL add 20 mg glycogen to 1 mL sterile molecular grade water.
8. Ethanol (70%): Each sample requires 250 µL. For 100 µL add 70 mL molecular grade ethanol to 30 mL sterile molecular grade water.
9. 1× Tris EDTA buffer (TE buffer): 10 mM Tris–HCl [pH 8.0], 1 mM EDTA. Each sample requires 50 µL. For 100 µL, combine 1 mL of 1 M Tris–HCl and 200 µL 0.5 M EDTA. Bring total volume to 100 mL using sterile molecular grade water.
10. Bench top mini-bead beater homogenizer (e.g., MagNA Lyser, Roche).

2.4 16S rDNA qPCR

1. PCR Mastermix: Several mixes are commercially available such as TaqMan® Universal PCR Master Mix, Applied Biosystems (Cat. Number: 4304437). Ensure that mix contains PCR buffer reagents (500 mM KCl, 100 mM Tris–HCl [pH 8.0], dNTP mix (2.5 mM each dNTP), 50 mM Magnesium Chloride, and Taq Polymerase.
2. SSU fluorogenic broad-specificity oligonucleotide primers for total bacterial 16S DNA (*see* **Note 1**).
3. 96-well PCR plate and 96-well plate cold block.
4. Plate sealing film for qPCR: e.g., ThermaSeal RT2RR™, Excel scientific (Cat. Number TS-RT2RR-100).
5. Standard 16S bacterial DNA template (*see* **Note 2**).
6. Mixed community genomic DNA (*see* **step 3**).
7. Thermocycler with Real-Time PCR capability.

2.5 Molecular Barcoding

1. Barcoding primers (*see* **Note 3**).
2. PCR Mastermix (Nova*Taq*™), 96-well PCR plate and 96-well plate cold block as described in Subheading 2.3, **item 1**.
3. Mixed community genomic DNA (*see* **step 3**).
4. Plate sealing film for PCR.
5. Thermocyler.

2.6 Agarose Gel Electrophoresis

1. Gel electrophoresis separation station and power supply.
2. TAE buffer (50× stock): 2 M Tris-Acetate, 0.05 M EDTA [pH 7.6]. Add 242 g Tris-base to 750 mL deionized RNAase free water in a 1 L graduated cylinder. Add 100 mL of 0.5 M EDTA to the solution and 57.1 mL glacial acetic acid. Adjust pH to 7.6 (at 25 °C) and bring final volume to 1 L. Filter solution through 0.22 μm filter to remove particles. Dilute to a 1× working solution when needed. (20 mL of 50× stock in 980 mL deionized RNAse free water).
3. Agarose.
4. Ethidium bromide: add 0.5 μg ethidium bromide per 1 mL agarose gel solution.
5. DNA Ladder (1 Kb).
6. Glycerol bromophenol blue loading dye (6×).
7. Long wavelength ultraviolet transilluminator.

2.7 DNA Normalization Reagents

1. SequalPrep® normalization plate kit, 96-well, Applied Biosystems (Cat. Number: A10510-01).

2.8 Sample Concentration and DNA Purification

1. Vacuum concentrator, e.g., Savant SpeedVac™ DNA Concentrator (Fisher).
2. Spin-column based DNA purification kit: e.g., DNA Clean & Concentrator™-5 (Zymo Research) or QIAquick PCR Purification columns (Qiagen).

2.9 Fluorometric DNA Quantification

1. Fluorometric DNA quantification kit: e.g., Qubit® dsDNA high sensitivity assay kit, Fisher (Cat. Number: Q32851) (*see* **Note 4**).
2. Fluorometer: e.g., Qubit® 3 fluorometer (Fisher).

3 Methods

Several commercially available DNA isolation kits such as the UltraClean® Microbial DNA Isolation Kit (Cat No: 12224-50) offered by Mo Bio Laboratories Inc. allow for efficient DNA extraction from swabs. Here we present two alternative protocols that may be used in addition to commercially available kits. Spin-column based kits use a solid-phase silica gel membrane that binds DNA when the pH and salt concentrations are optimal. This is easily saturated when samples contain large amounts of genomic DNA. The following two methods do not use solid phase sorbents and therefore are scalable allowing for isolation of larger amounts of genomic DNA. Each method presented below has been shown to yield comparable results [20, 22]. We recommend solvent-based phenol–chloroform extraction with mechanical bead-beating for samples with large amounts of tissue or blood (*see* Table 1 for examples of concentrations and Table 2 for examples of purity measurements obtained). Ensure that appropriate negative and positive controls are included in the DNA isolation. Sterile swabs should be extracted alongside patient samples to serve as negative controls. A positive control can be created by swabbing an agar plate containing bacterial colonies.

In order to reduce nucleic acid contamination, conduct all DNA isolation steps and PCR amplification preparation within a HEPA filtered laminar flow hood. Ensure that all surfaces are wiped clean with 70% ethanol and disinfect the hood with 15 min of ultraviolet light before beginning.

3.1 Swab Collection and Storage

1. Sample sinonasal mucosa by rotating swab clockwise at least five times in the region of interest, until the swab is visually saturated. Transfer swab to original collection tube and store on ice (*see* **Note 5**).
2. Within 2 h of collection, transfer the swabs to a sterile 2 mL screw cap microcentrifuge tube in a disinfected laminar flow hood. The excess swab may be trimmed using isopropanol-treated scissors or carefully snapped off to allow the microcentrifuge tube lid to close.
3. Store swabs at −80 °C until further processing.

Table 1

An overnight culture of methicillin-resistant *Staphylococcus aureus* (MRSA) in tryptic soy broth was divided into identical 1 mL aliquots and subjected to various conditions for DNA extraction in duplicate: Negative (no bacteria), Vortex (vortex only), Boil (vortex and boil only), and bead beat at 3000 rpm (845 × *g*), 5000 rpm (2348 × *g*), or 7000 rpm (4602 × *g*) vs. 30, 60, or 99 s on a Roche MagNA Lyser bead beater[a]

DNA concentration (ng/μL)						
				Speed (rpm)		
Negative	Vortex	Boil		3000 (845 × *g*)	5000 (2348 × *g*)	7000 (4602 × *g*)
4.9	101.3	187.4	30 s	180.8	184.8	205
−22.8	107.3	180.8	30 s	175.6	183.6	210
			60 s	202.9	176.7	224.6
			60 s	188.6	194	213.1
			99 s	249.9	213.2	242.1
			99 s	240.9	234.5	231.4

After extraction each sample was centrifuged for 5 min at 10,000 rpm (9391 × *g*) and the supernatant containing DNA was saved. DNA concentrations (ng/μL) were measured using a NanoDrop ND-1000 spectophotometer

[a]Samples subjected to bead beating were also vortexed and boiled beforehand

Table 2

DNA purity (from the samples described in Table 1) was determined by measuring the ratio of absorbance at 260 nm and 280 nm using a NanoDrop ND-1000 spectrophotometer

Absorbance ratio 260/280 nm						
				Speed (rpm)		
Negative	Vortex	Boil		3000 (845 × *g*)	5000 (2348 × *g*)	7000 (4602 × *g*)
−0.23	1.42	1.71	30 s	1.83	1.82	1.77
0.46	1.43	1.75	30 s	1.86	1.84	1.71
			60 s	1.79	1.84	1.68
			60 s	1.85	1.79	1.79
			99 s	1.76	1.76	1.81
			99 s	1.76	1.76	1.71

A 260/280 ratio of ~1.8 is generally considered "pure" for DNA

3.2 DNA Isolation of Low Biomass Swabs

1. Add 500 μL of TEN buffer to the dried swab heads in the same 2 mL screw-cap tube used for storage.
2. Place tubes in 95 °C incubator bath for 10 min to lyse cells. After 10 min, remove each sample and vortex for 30 s. Incubate tubes at 95 °C for an additional 10 min.

3. Remove swabs using isopropanol-treated forceps and place samples on ice or store at −20 °C until PCR.
4. If additional extraction is desired, add 0.25 g of 0.1 mm zirconium beads and bead beat for 60 s at 6000 rpm (3381 × *g*) (*see* **Note 6**).
5. Centrifuge the samples at 10,000 rpm (9391 × *g*) for 5 min and transfer supernatant to a clean 2 mL screw-cap tube. Samples can be stored at −20 °C until use.

3.3 DNA Extraction of High Biomass Swabs

1. Add 700 µL of 2× buffer B and 0.25 g of 0.1 mm zirconium beads to each specimen tube (*see* **Notes 7** and **8**).
2. Mix by inverting several times.

3.3.1 Cell Lysis

3. Add 500 µL phenol–chloroform to each sample (*see* **Note 9**).
4. Place samples in a mini bead-beater homogenizer for 60 s at 6000 rpm (3381 × *g*). At the completion of the bead-beating cycle, the cotton tip of each swab and any visible tissue should be completely homogenized.
5. Centrifuge samples at 10,000 rpm (9391 × *g*) at room temperature for 5 min to deposit beads and sample debris into pellet.
6. Two phases (an upper aqueous phase and a lower organic phase and interface) will partition following centrifugation. Collect the upper aqueous phase using a pipette and transfer to a fresh 2 mL tube.
7. Repeat this process until the interface (thin area between the upper aqueous and lower organic phase) is clear. Collect only the upper aqueous phase and transfer to a fresh 2 mL tube.

3.3.2 Precipitation and DNA Isolation

8. Add 300 µL 7.5 M ammonium acetate, 500 µL 100% isopropanol, and 2 µL of 20 mg/mL glycogen (*see* **Note 10**).
9. Incubate samples at −80 °C for 2 h (*see* **Note 11**).
10. Centrifuge samples at 10,000 rpm (9391 × *g*) for 30 min at 4 °C.
11. Decant supernatant by drawing off liquid from each sample using a pipette without disrupting the pellet. Discard supernatant. Not all samples will have a visible pellet.
12. Add 250 µL of cold 70% ethanol to each sample. Vortex briefly and centrifuge for 1 min at 10,000 rpm (9391 × *g*). Carefully remove supernatant without disrupting pellet. Discard supernatant (*see* **Note 12**).
13. Evaporate solvent to dryness in a vacuum concentrator (e.g., SpeedVac™, Thermo Scientific) or if unavailable, samples may be evaporated in a UV-sterilized laminar flow hood.
14. Resuspend pellets in 50 µL of 1× TE buffer. Tightly close the microcentrifuge tubes prior to storage to limit evaporation. Concentrated DNA Samples can be stored at −80 °C.

3.4 Measuring Total Bacterial 16S DNA by qPCR

1. Prepare the mastermix in a 2 mL microcentrifuge tube on a cold block. The total PCR reaction volume is 20 μL per sample. Use the appropriate dilution of master mix stock according to the manufacturer. For example, aliquot 10 μL 2× master mix, 7 μL of sterile RNAase free water, and 1 μL of 20× forward and reverse primers. Prepare sufficient excess mastermix to allow for appropriate controls including negative controls (DNA extracts from sterile swabs and RNAase-free water alone), positive extraction controls (bacterial colonies swabbed from agar), and standard curve wells.
2. Pipette 18 μL of the mastermix into a corresponding well in a 96-well plate on a cold block.
3. Add 2 μL of extracted genomic DNA from each swab sample to its corresponding well on the 96-well plate.
4. Add appropriate amount of genomic or plasmid based 16S bacterial DNA template to create standard curve ranging from 10^8 to 10^1 copies (*see* **Note 2**).
5. Cover the plate tightly with qPCR compatible plate sealing film, vortex briefly, and centrifuge plate briefly to ensure sample is completely mixed.
6. Transfer 96-well plate to thermocycler and initiate qPCR with 7-min denaturation at 95 °C followed by 40 cycles of 15 s at 95 °C, and 1 min at 60 °C, including a plate-reading step at the end of each cycle.
7. After the qPCR run completes, use cycle threshold values (C_t) to determine the appropriate number of cycles necessary to amplify DNA in the subsequent barcoding step (*see* **Note 13**). *See* Table 3 for example C_t values obtained.

3.5 Molecular Barcoding

1. Aliquot 20× forward and reverse barcoding primers into 96-well plate with each well containing a unique barcoded sequence with the same 5′ sequencing platform adapter oligonucleotide and the 16S DNA universal primer sequence. Document the location of each barcode on the 96-well primer plate. Cover the primer plate with aluminum sealing foil and store at −20 °C when not in use.
2. Prepare the mastermix (Nova*Taq*™) for the barcoding reaction in a 2 mL microcentrifuge tube on a cold block. The total PCR reaction volume is 30 μL per sample. Use the appropriate dilution of master mix stock according to the manufacturer as performed in Subheading 3.4, **step 1**. Distribute the master mix (without primers) and sterile water into corresponding wells on a new 96-well plate on a cold block.
3. Using a multichannel pipette, transfer 1.5 μL of each forward and reverse barcoding primers from the 96-well primer plate to

Table 3

Cycle threshold (C_t) values for qRT-PCR of the total bacterial 16S gene for DNA samples (described in Table 1)

16S qRT-PCR C_t Values						
				Speed (rpm)		
Negative	Vortex	Boil		3000 (845 × *g*)	5000 (2348 × *g*)	7000 (4602 × *g*)
36.35	31.25	30.87	30 s	28.21	21.14	19.41
36.94	28.72	32.7	30 s	28.24	24.25	19.1
			60 s	21.57	17.15	15.39
			60 s	21.7	17.42	16.13
			99 s	17.28	16.39	15.17
			99 s	18.05	16.02	15.36

A lower C_t value correlates with a higher concentration of bacterial DNA. (Negative controls for the PCR reagents did not contain any detectable levels of bacterial DNA)

the corresponding wells on the sample plate. Prepare sufficient excess mastermix to allow for appropriate controls including negative controls (DNA extracts from sterile swabs and RNAse-free water alone) and positive extraction controls (bacterial colonies swabbed from agar).

4. Add 4 μL of mixed-community genomic DNA extract from each swab sample to its corresponding well on the 96-well sample plate.
5. Cover the 96-well sample plate with an adhesive plate seal, briefly vortex, and centrifuge to concentrate sample in the bottom of each well.
6. Transfer 96-well plate to thermocycler and initiate PCR with 7-min denaturation at 95 °C followed by 30 s at 94 °C, 30 s at 54.3 °C, and 60 s at 72 °C for the appropriate number of cycles. Following the last cycle, initiate a final extension of 10 min at 72 °C. Determine the optimal number of cycles based on previous C_t results (*see* **Note 13**). Typical cycles used for barcoding sinus samples range between 26 and 30.
7. After the thermocycling sequence is complete, plate may be stored at −20 °C.

3.6 Confirmation of Amplification with Gel Electrophoresis

8. Since florescent probes would interfere with the sequencing process, the best way to ensure sample 16S gene amplification is with an agarose DNA gel. Prepare a 2% agarose gel by mixing agarose with the appropriate volume of 1× TAE buffer for the gel cassette you will be using. For example, mix 2 g agarose in 100 mL 1× TAE buffer.

9. Microwave the mixture for 1–3 min until the agarose is completely dissolved and the solution is gently boiling. Carefully remove the solution from the microwave and let cool on the bench top for 5 min.
10. Add ethidium bromide to the mixture to a final concentration of approximately 0.2–0.5 μg/mL.
11. Pour the gel into the gel tray and position the well comb in place. Allow gel to set at 4 °C or room temperature until completely solidified.
12. Place gel into gel box and completely submerge in 1× TAE buffer. Remove well comb (*see* **Note 14**).
13. Add 1 Kb DNA molecular weight ladder to the first well on agarose gel.
14. Mix 10 μL of loading buffer with 5 μL of barcoded DNA and transfer to well on agarose gel. Repeat for all barcoded samples. Note the position of each sample on the gel relative to its corresponding location on the 96-well plate.
15. Run the gel at 100 V until the dye migrates 75–80% through the gel. Turn off power supply and remove gel.
16. Visualize DNA bands using long-wavelength ultraviolet light (*see* **Note 15**).

3.7 DNA Concentration Normalization and Sample Pooling

1. Load the remaining 25 μL of amplicon from each sample in the barcoding reaction plate into a corresponding well on a SequalPrep™ 96-well normalization plate (*see* **Note 16**).
2. Follow protocol for DNA binding, washing, and elution steps as noted in the SequalPrep® normalization plate kit protocol such that each sample is suspended in 20 μL of elution buffer.
3. Pool all samples into a clean 2 mL microcentrifuge tube and transfer to vacuum concentrator. Evaporate until ~200 μL elution buffer remains. Large sample sets may require more than one microcentrifuge tube to concentrate DNA. Concentrated pooled amplicons may be stored at −80 °C.

3.8 Purify and Measure DNA Concentration

1. Transfer 100 μL pooled and concentrated amplicons to a clean 2 mL microcentrifuge tube. Follow Clean & Concentrator™-5 kit (or similar kit) instructions. Elute sample in 30 μL of DNA elution buffer (*see* **Note 17**).
2. Follow fluorometric DNA assay kit protocol to determine concentration of purified, pooled and barcoded amplicon sample and dilute DNA to the appropriate concentration for the specific sequencing platform being used.

4 Notes

1. Primers for total bacterial DNA qPCR are generated using amplicons of the 16S rRNA gene with fluorogenic reporters (such as HEX or FAM) as described by Nadkarni et al. [32]. There are 9 hypervariable regions contained within the bacterial 16S rRNA gene. Primers that target specific segments of the variable region may preferentially target certain taxa with variability contained in the selected region [25]. There are several examples in the literature of 16S rRNA amplification from upper respiratory swabs using primers that target the variable regions V1-V3 for 16S rRNA genes 8F, 805R, 515F, and 1391R among others [20, 22, 33].
2. In order to perform qPCR on mixed-community DNA extracted from swab samples, a standard curve must be generated with known amounts of bacterial gene template. Several approaches have been reported using genomic DNA or DNA derived from plasmids to generate bacterial DNA templates for qPCR. We utilize a plasmid vector to produce Lachnospiraceae 16S gene template and construct standard curves ranging from 10^8 to 10^1 copies by making tenfold serial dilutions of the template stock.
3. Barcoding primers are similar to the universal primers designed to amplify the 16S rRNA gene. However, barcoding primers contain short oligonucleotide sequences that are unique to an individual sample that are later used to identify the sample after all samples are pooled for sequencing. Additionally, barcoding primers are designed to contain platform-specific 5′ adapter oligonucleotide sequences that are specific to the sequencing platform being used. Each sequencing platform provides detailed amplicon sequencing protocols that provide the adaptor sequences. There are several software programs that facilitate the efficient design of barcoding primers. For example, BARCRAWL and BARTAB are open source software tools available for designing barcoding primers [23, 24].
4. Many of the sequencing platforms currently in use are exquisitely sensitive to DNA overloading. Therefore, it is important that a reliable and accurate method is used to quantify the DNA prior to loading into the sequencer. Traditional methods using absorbance ratios at 260 nm and 280 nm (A_{260}/A_{280}) are not sensitive at very low concentrations of DNA, and absorbance varies with pH. Using a fluorometric method for DNA quantification allows for a more specific and sensitive measurement. There are several commercially available fluorometric kits that utilize Hoechst 33258 or PicoGreen.
5. Since the bacterial niche is variable from different sites within the nose and paranasal sinuses, it is critical to sample the spe-

cific region of interest using a consistent and standardized technique. In clinical settings where multiple clinicians are collecting swabs, standard collection protocols should be used. Sinus samples that come in contact with the mucosa of the anterior nares upon removal should be discarded.

6. When using a bead beater to extract DNA, the conditions should first be optimized in order to determine the most appropriate length and speed that extracts the most DNA without damaging it. An easy way to do this is to prepare several identical 1 mL aliquots of the same overnight culture in liquid media, pellet the cells by centrifugation, and resuspend in 1× TEN with 0.1 mm zirconia beads. Boil as previously instructed and then bead beat at different combinations and variations of time and speed in replicates. Pellet the debris by centrifugation and save the supernatant containing genomic DNA. Optimal conditions are determined by measuring the optical density at 260 nm for concentration, recording the OD_{260}/OD_{280} ratio for purity, and deciphering which condition yields the best Ct values.
7. In order to ensure lysis of all bacterial cells, some protocols add lysozyme to the buffer B (final concentration 5 mg/mL) and incubate for 30 min at 37 °C to help disrupt gram-positive cell walls. This is followed by the addition of SDS (final concentration 0.3%) and proteinase K (final concentration 2 mg/mL) and further incubation at 50 °C for 30 min prior to bead homogenization.
8. Zirconia/silica beads are spherical and do not fracture under extremely high shearing forces. These will effectively shear cell walls but leave the DNA intact.
9. Phenol–chloroform is a mixture of organic solvents that trap non-nucleic acid components of lysed cells such as lipids, proteins, and polysaccharides.
10. This step directly leads to the precipitation of DNA from the solution. DNA is insoluble in isopropanol. Isopropanol precipitates DNA at much lower concentrations compared to other alcohols such as ethanol. Ammonium acetate is added to increase the monovalent cation salt concentration and aids in precipitation of DNA and decreases precipitation of free nucleotides. Glycogen is a large polysaccharide that is added as an inert carrier (co-precipitant), which helps trap nucleic acids by bulk to increase DNA recovery during centrifugation—especially in swab samples containing very low concentrations of DNA.
11. Precipitation of DNA at lower temperatures slows the precipitation by increasing the dielectric constant and the viscosity of the mixture. Decreasing the temperature helps DNA retain a native shape and limits the actions of endogenous nucleases

during the precipitation. However, the tradeoff of performing precipitations at lower temperatures is lower DNA yields and some co-precipitation of salt components.

12. Washing the pellet with 70% ethanol helps remove any salts that co-precipitated with the genomic DNA.
13. The cycle threshold (C_t) is the number of cycles necessary for the fluorescence in the qPCR sample to exceed the background level. Lower C_t values reflect higher levels of 16S amplification within a sample. Sinus swabs often yield abundant 16S DNA with typical C_t values ranging from 12 to 30. After the completion of the qPCR run, samples with lower C_t values must be diluted to normalize the concentration across samples prior to barcoding. For example, if the mean C_t value for a group of sinus swab samples is 25, samples with C_t values of 12 must be diluted 1:2 in order to more closely match the amount of 16S DNA contained in the remaining samples. Samples with C_t values of 34 or higher typically do not contain amplified DNA beyond background levels. Samples with C_t values greater than the mean should proceed to barcoding without concentrating. The minimum number of cycles should be used to maximize the variability of amplified DNA. The qPCR run can also be used to determine the optimal number of cycles for subsequent barcoding, which for sinus swab samples is typically 26–30 cycles.
14. Ethidium bromide (EtBr) is a DNA intercalating agent and known mutagen. Use appropriate PPE when working with EtBr and dispose of waste properly. Adding EtBr allows for the DNA bands to be visualized using ultraviolet light. EtBr is also positively charged and therefore tends to run in the opposite direction of DNA. In order to minimize the separation of EtBr from DNA bands, add a small amount of EtBr to the running buffer (approximately 0.1 μg/mL).
15. Normally two bands are visualized on the agarose gel including a single band corresponding to the length of the target amplicon (length of forward primer minus length of reverse primer). The lowest molecular weight band (furthest migrated through the gel) is composed of primer dimers and loading buffer components. Occasionally, there is a higher molecular weight band that separates above the desired sequence. This reflects nonspecific binding of the primers to off-target genes. If the nonspecific binding band is substantial, an additional gel purification step may be added wherein the band containing the desired DNA is carefully cut out from the gel and purified. Samples that do not show an amplified band corresponding to 16S DNA should correspond to DNA extracts with high C_t values in the total bacteria qPCR run. Samples with low C_t values by qPCR that subsequently do not amplify in the barcoding PCR reaction should be troubleshooted and repeated.

16. Several methods for DNA normalization are available including direct quantification (using NanoDrop technology), size restricted DNA quantification (such as the QIAxcel from Qiagen), and quantitative DNA binding (such as SequalPrep kit from Invitrogen). The SequalPrep kit has been shown to produce the best normalized barcoded amplicon pool and offers a more efficient workflow than other alternatives [34].
17. The barcoding PCR reaction products contain primer dimers, salts, enzymes, and other impurities. Although the SequalPrep normalization step removes several of these impurities, there may be primer dimers and cationic salts that remain bound to the DNA. Therefore, we implement a quick spin-column based purification step prior to sequencing.

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